SEX DIFFERENCES AND ENVIRONMENTAL INFLUENCE ON DOPA-OXIDIZING ACTIVITY IN DROSOPHILA MELANOGASTER [1]

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Horowitz and Fling (1955) have described a rapid method for determination of tyrosinase activity in Drosophila. The method involves grinding adults, centrifuging, and decanting the supernatant as enzyme source. Colorimetric readings of the dopa-oxidizing activity are made to obtain enzyme-substrate reaction curves. The authors found that the maximal rate of dopachrome formation at 30° C. is directly proportional to enzyme concentration. This rapid method was used in a series of studies by the writer because it made possible quick repetition of determinations and thereby allowed for on-the-spot analysis of dopa-oxidizing activity during metamorphosis. (The exact nature of the enzymes involved is not known. Danneel (1943) has described three phenolases in Drosophila: one, a monophenolase which converts tyrosine to dopa, another a diphenolase which converts dopa to dopachrome, and a third which converts dopachrome to melanin.)

In attempting to apply this method, the writer obtained very different activity levels among 50-mg. samples of larvae and adults, even when these were taken from the same batch. It was found that modification of the method (as described below) eliminated some of the variation in activity rates between different samples. However, much variation still remained, and it was suspected that sex of the adults and larvae, and exposure of larvae to relatively dry air of the air conditioned laboratory were influencing the subsequent rate of dopa-oxidizing activity. The following study was undertaken to determine whether these factors do influence the rate of activity.

Materials and Methods

The flies used were those collected from a wild population at Beaufort, N. C. (see Jacobs, 1960). The strain is a light tan form, similar to Canton-S, made "isogenic" for chromosomes 1, 2, and 3 by means of a dominant inversion j90 marker stock from Dr. H. J. Muller and a C1B stock from Dr. M. Whittinghill.

The culture medium used was a modification of the Carpenter-Baker formula containing: 1000 cc. water, 40 g. white ground cornmeal, 15 g. agar, 40 g. dried Fleischmann's yeast type 2019, 1 g. KH_2PO_4, 8 g. Rochelle salt, 100 cc. white Karo syrup, and 0.5 g. of: NaCl, $CaCl_2$, $MnCl_2$ and $FeSO_4$. The medium was seeded with an aqueous suspension of dry active Fleischmann's yeast.

About 50 cc. of medium were placed in each half-pint milk bottle. To such

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bottles were added 30 female and 10 male parents. Each day, the parents were transferred to a fresh bottle to which ten fresh females and two males were added.

All cultures were kept at 25° C. in the dark. When the late larvae crawled up the sides of the bottles, as they do before pupation, the largest were removed and sexed, a few at a time, under water, on the basis of gonad size (see Bodenstein, 1950). Emergence tests of some of these larvae showed that the reliability of the sexing technique was excellent. For each enzyme determination, approximately 200 of the sexed larvae were placed in each of two 55 × 55 mm. specimen jars. One of these jars was placed, without a lid, in a 250-mm. Scheibler desiccator in which a saturated solution of $K_2CO_3 \cdot 2H_2O$ was kept in order to produce 43% R. H. (relative humidity) conditions (see Lange, 1939). The other jar was fitted with a lid with a hole 45 mm. in diameter cut in it and a disc of wire gauze fit in it, to prevent larvae from crawling out of the jar, which is possible when the walls are wet. This second jar was placed in a desiccator containing distilled water. The two desiccators were placed in the dark in a G. E. refrigerator kept at 24.5° C. with a Cenco 46042 heater thermostat unit. After the desiccators had stood for four hours, 50 mg. of the largest larvae (to the nearest whole larva) were used for each enzyme determination.

Adults were obtained by etherizing them within an hour after emergence from the pupa cases, while they were still translucent and just after expansion of the wings. Before the adults were used for the colorimetric enzyme determinations, the heads were removed so the eye pigments would not interfere with the tests. It took about five minutes to remove the heads from the 50 mg. of flies used in each determination. The flies were weighed before removal of the heads.

The method for dopa-oxidizing activity was as follows: from the 100% R. H. desiccator were weighed 50 mg. of larvae (to the nearest whole larva). These were washed with distilled water into a powder funnel, to the bottom of which was fitted a wire gauze. The funnel was then inverted over the open end of a 13 × 100 mm. Corning #7725 tissue grinder tube sawed off to a length of 45 mm. so it would fit the high speed head of the centrifuge. Distilled water was streamed onto the wire gauze to wash the larvae into the grinder tube. This water was pipetted out and replaced three times, after which all free water was pipetted from the inside of the tube, and the outside was wiped dry. The tube with the larvae was then weighed. The larvae were ground in the tube in 1 ml. of phosphate buffer (pH 6). To the tube was immediately added 0.5 ml. of L-dopa (0.02 *M* aqueous), and the tube was placed in a water bath at 30° C. for ten minutes. The tube was then placed into ice water for one minute while 1 ml. of chloroform was added. The tube was then removed, Parafilm was held over the open end, and the tube was shaken a few seconds so the lipids would dissolve in the chloroform, for lipids were found to cause turbidity which interfered with colorimeter readings. The tube was then placed into the high speed head of the centrifuge (0° C.) and brought to 6,000 *g* in one minute, left there for five minutes, then reduced to stopping in one minute. Into a ½-inch tube of the B & L Spectronic 20 colorimeter were placed 0.5 ml. of the supernatant and 2 ml. of the phosphate buffer. A reading was taken against a distilled water standard at 475 millimicra, and the tube was placed in the water bath at 30° C. for ten minutes, after which another reading was made.

An equal number of larvae were removed from the 43% R. H. desiccator and treated as were the 100% R. H. larvae except that after the tube with the larvae was weighed, water was added to bring the total weight up to that shown by the tube with the 43% larvae. (In the study of unsexed larvae kept under the two conditions as described in the next paragraph, in which the larvae were never in water, the 43% R. H. larvae were found to weigh an average of 0.833% less than the 100% R. H. larvae.) Adults were added unwashed to the grinder tube, ground in buffer, and treated the same way as were the larvae.

When it was found that larvae at 43% R. H. showed increased dopa-oxidizing activity as compared with those at 100%, the question arose as to whether the 43% larvae would also pupate at a faster rate. To test this, 200 unsexed larvae were placed in the jar in each desiccator and allowed to remain for four hours as before, after which 20 of the largest larvae in each desiccator were weighed, boiling water was poured into the jars, and the pupae were counted. This was repeated ten times for the four-hour period and ten times for an eight-hour period.

Results

The results of the tests showed larvae kept at 43% R. H. had greater dopa-oxidizing activity than those kept at 100% (Table I). For the larvae at 43%,

Table I

Dopa-oxidizing activity of Drosophila melanogaster *as indicated by colorimeter readings of reactant mixtures of tissue homogenates and dopa substrate. A low per cent of transmittance indicates a high rate of dopa-oxidizing activity*

Type of flies	Number of 50-mg. samples	Per cent of transmittance		
		Reading no. 1 (after centrifuging) Mean ± S.E.	Reading no. 2 (10 min. after no. 1) Mean ± S.E.	Difference between no. 1-no. 2 readings Mean ± S.E.
Larval females in humid conditions (100% R. H.)	15	70.86 ± 2.42	59.66 ± 3.47	11.20 ± 1.63
Larval females in dry conditions (43% R. H.)	15	60.00 ± 2.06	42.40 ± 3.04	17.60 ± 0.92
Larval males in humid conditions (100% R. H.)	13	76.07 ± 2.77	66.61 ± 2.45	9.46 ± 1.83
Larval males in dry conditions (43% R. H.)	13	66.38 ± 3.58	51.61 ± 3.97	14.77 ± 1.58
Adult females	53	59.77 ± 0.97	51.67 ± 1.24	8.10 ± 0.52
Adult males	38	85.60 ± 0.23	84.00 ± 0.34	1.60 ± 0.71

the colorimeter readings taken just after centrifuging, as well as those taken ten minutes later, showed lower per cent of transmittance than did those for the larvae kept at 100% R. H. Since this drop in per cent of transmittance is sufficient to show the relative activity rate, this figure alone will be considered in this discussion for the sake of simplicity, and will be referred to as activity in colorimeter units.

Larval females at 100% R. H. showed 11.20 units, while those at 43% R. H. showed 17.60 units of activity. Larval males at 100% R. H. showed 9.46 units, while those at 43% showed 14.77 units. Adult females showed 8.10 units, while adult males showed only 1.60 units. It is thus observed that the larvae at 43% R. H. showed greater activity than those at 100%, and adult females showed greater activity than adult males. All these differences are significant well below the 5% level. It appears that larval females show greater activity than larval males, but the observed "t" value for this difference is small (1.52) and above the 5% level of significance.

For both larvae and adults, the average wet weight of females was greater than that of males (Table II). It took an average of only 25.52 larval females to weigh 50 mg., while it took 30.53 males. Likewise, it took only 39.87 adult females, while it took 55.14 males.

In the counts of larvae kept at 100% and 43% R. H. for four hours, there were 381 pupae in the 100% jars and 352 in the 43% jars. The chi-square value for this difference is only 1.147, which is not significant. More significant results were obtained in the eight-hour samples, in which 674 pupae were counted in the

TABLE II

Mean number of flies in 50-mg. samples taken to the nearest whole fly. The same number of larvae were used in the 100% and 43% relative humidity samples

Type of flies	Number of 50-mg. samples	Number of flies per 50-mg. sample (Mean ± S.E.)
Larval females	15	25.52 ± 0.06
Larval males	13	30.53 ± 0.05
Adult females	53	39.87 ± 0.07
Adult males	38	55.14 ± 0.09

100% jars and only 594 in the 43% jars. The chi-square value for this difference is 50.473, which is significant below the 1% level. Thus, contrary to what was expected, the larvae at 100% R. H. pupated at a faster rate than those at 43% R. H.

DISCUSSION

The observation that *D. melanogaster* females show more dopa-oxidizing activity than males raises the question as to whether this activity is involved in general metabolism, perhaps as a result of egg production. This would seem reasonable in view of the fact that differences in gonad size, as observed among male and female larvae, are less than the difference in size between the reproductive structure (egg masses) of the adult female as compared with the small mass of the adult male testes: and difference in dopa-oxidizing activity among male and female larvae is also smaller than the difference in activity between male and female adults. Bodine and Allen (1938) have demonstrated, in grasshopper eggs, a tyrosinase proenzyme which can be activated by shaking and other means. Kucera (1934) has found *D. melanogaster* adult females to consume more oxygen than adult males. However, Sussman (1952) found no difference in oxygen uptake in *Platysamia cecropia* moth diapausing larvae injected with tyrosinase inhibitors, as compared with controls, and he suggested that tyrosinase is not a terminal oxidase in those larvae.

The increased rate of dopa-oxidizing activity in the larvae at 43% R. H. might be due to control by Weismann's ring gland, which in turn is regulated by the nervous system. Dennell (1949) has inhibited tyrosinase activity in *Calliphora erythrocephala* by destruction of the gland. In this fly, in spite of the presence in the blood of both enzyme and substrate, no oxidation of tyrosine takes place until the pupation hormone controlled by the gland is liberated. Dennell discovered a glucose dehydrogenase system which declined in activity as the larvae approached pupation, while tyrosinase activity increased. Dennell suggests that the dehydrogenase system is controlled by the gland and inhibits tyrosinase activity.

The classical view of the role of phenolase activity in insects is that certain phenolic oxidation products of tyrosine are responsible for tanning and hardening of the larval cuticle to form the puparium. (See Dennell, 1958a, for review.) It was this theory which prompted the present study in which pupae were counted following killing with boiling water. It was thought that the increased phenolase activity would cause increased rate of puparium formation. But, contrary to what was suspected, the larvae at the lower humidity (43%), in which dopa-oxidizing activity was accelerated, showed a slower pupation rate than those at 100%. This decreased rate at lower humidity has not yet been explained.

The failure to find an increase in pupation rate to accompany an increase in dopa-oxidizing activity in larvae at the lower humidity is in accordance with the finding of Dennell (1958b) that inhibition of tyrosinase by injection of phenylthiourea into mature larvae of the fly, *Calliphora vomitoria*, did not modify puparium formation. Dennell (1958a) has been led to the view that the dihydroxyphenol formed in the blood of insects by the specific action of tyrosinase on tyrosine is not of primary importance in giving rise to a tanning quinone. He thinks this activity is of importance only in the formation of melanin, although he admits the possibility that some dopa formed in the blood may be hydroxylated in the cuticle to yield a slight supply of the tanning quinone in addition to that produced by the major source. Dennell thinks the major source of the tanning quinone arises from non-enzymatic hydroxylation of phenylalanine and tyrosine to hydroquinone, which is converted to para-benzoquinone which tans the cuticle. Malek (1957) also observed that sclerotization and melanization are two separate processes in the desert locust.

Ohnishi (1954a) found that ebony larvae of *D. melanogaster*, in which the pupa case is lighter than in the wild type, showed less tyrosinase activity than did the wild type. He also found (1954b) in larvae of ebony *D. virilis*, in which the pupa case is darker than in the wild type, that tyrosinase activity was higher than in the wild type. Ohnishi (1953) also found tyrosinase activity in *D. melanogaster* larvae increases five to six hours before puparium formation, that is, nearly at the critical period of pupation hormone. These observations lead Ohnishi to the view that tyrosinase is responsible for the color of the pupae. However, Danneel (1943) found no difference in tyrosinase activity among ebony, yellow, black, and wild type *D. melanogaster*, but he did note varietal differences. Karlson and Schmid (1955) found a metamorphosis hormone in Calliphora larvae to cause browning of the cuticle, and this browning was said to be due to tyrosinase, which the authors extracted. Much additional work is needed before this question of the role of tyrosinase and its genetic control in insect metabolism can be elucidated.

Summary

1. Colorimetric determinations of dopa-oxidizing activity in newly emerged adults and late larvae close to pupation time showed adult *Drosophila melanogaster* females to have higher activity rates than adult males. This may also be true for larvae, but the difference here was slight. The larvae showed higher rates than adults.

2. Larvae kept four hours at 43% relative humidity (R. H.) showed higher rates of dopa-oxidizing activity than did those kept at 100% R. H.

3. Contrary to what was expected, larvae kept at 100% R. H. pupated at a faster rate than those kept at 43%.

4. Female larvae and adults were found to be heavier than males.

5. The postulated role of tyrosinase in puparium formation is discussed.

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